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(54) Title: INDUCTION AND ENHANCEMENT OF THE IMMUNE RESPONSE TO TYPE-2 T CELL-INDEPENDENT ANTIGENS CONJUGATED TO LIPID OR LIPID-CONTAINING MOIETIES (57) Abstract The present invention provides a method of promoting a vigorous immune response to Type-2 T cell-independent antigens, such as bacterial polysaccharides, by the administration of a composition comprising a Type-2 T cell-independent antigen conjugated to a lipid or lipid-containing moiety, preferably, Lipo OspA. The composition promotes extensive class switching and memory to Type-2 T cell-independent antigens in immunocompetent and T cell-deficient hosts.		

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Induction and Enhancement of the Immune Response to Type 2 T Cell-independent Antigens Conjugated to Lipid or Lipid-containing Moieties

GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed and used for governmental purposes without the payment of any royalties to us thereon.

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application Serial No. 08/568,342, filed December 6, 1995, which is a continuation-in-part of application Serial No. 08/472,640, filed June 7, 1995, which is a continuation-in-part of application serial No. 08/422,830, filed April 17, 1995, all of which are specifically incorporated by reference.

FIELD OF THE INVENTION

The present invention relates to the use of lipids or lipid-containing compounds in inducing humoral immunity in response to polysaccharide antigens and other Type 2 T cell-independent antigens.

BACKGROUND

The cellular basis for induction of T cell-independent (TI) humoral immunity to bacterial organisms and their antigenic constituents is largely unknown, thus hampering efforts to develop sufficient defenses against bacterial infection. This poses serious problems, given the prevalence and significance of Type-2 T cell independent (TI-2) antigens from bacterial organisms such as *Haemophilus influenzae* type b polyribosyl-ribitol-phosphate (PRP), Pneumococcal capsular polysaccharides (including type III), Group B Streptococcus, *N. meningitides*, *salmonella*, and *P. aeruginosa* mucoexopolysaccharides, including *P. aeruginosa* Fisher type 1 capsular polysaccharides.

Antigens are generally classified in the art as either T-dependent (TD) or T-independent (TI) antigens. In addition, haptens are small molecules which, though not antigenic themselves, can be recognized by preformed

antibodies and can stimulate an antigenic response if coupled to a T-dependent or T-independent carrier. The general properties and characteristics of these antigens are well known in the art and have been described in Roit, Essential Immunology, Blackwell Scientific Publications (1994), and Paul, Fundamental Immunology, Raven Press (1989), each of which is incorporated herein by reference.

T-dependent antigens (which include most proteins) contain both T and B cell epitopes and can stimulate the production of antibodies through the Class II MHC pathway. Briefly, this entails the uptake, processing and cell surface expression of T-dependent epitopes by antigen presenting cells, generally dendritic cells, macrophages, and B cells.

The macrophage or dendritic cell engulfs the T-dependent antigens in a pinocytotic vesicle, where it is degraded into short fragments by endosomal proteases. Antigen fragments bind to newly synthesized Class II Major Histocompatibility Complex proteins (MHC) and are subsequently exported to the cell surface as part of a Class II MHC-peptide complex. In this context, the macrophage or dendritic cell presents the antigen fragments to T cells; these T cells in turn stimulate those B cells which have also interacted with that particular antigen.

Similarly, B cells expressing surface IgM or IgD specific for a particular antigen capture and internalize that molecule through receptor-mediated endocytosis. At this point, the B cell itself acts as a Class II antigen presenting cell, processing the T-dependent antigen into peptides and re-expressing the fragments on the cell surface, and in the context of Class II MHC. T cells recognizing the processed peptides on the B cell surface (and in the context of Class II molecules) directly stimulate antibody production in the presenting B cell.

Unlike TD antigens, T-independent antigens stimulate B cells to secrete antibody without the need for direct T cell involvement. These TI antigens are further classified as Type 1 (TI-1) and Type 2 (TI-2) based on

their method of binding to the B cell, their intrinsic B cell activating properties and subsequent immunological effects.

The TI-2 class of antigens are linear antigens which are not readily degraded in the body and which have regularly spaced, highly repeating determinants. TI-2 antigens commonly comprise large polysaccharide polymers such as those derived from bacterial cell walls or flagella. Other common examples of TI-2 antigens include FICOLL, D-amino acid polymers, polyvinylpyrrolidone, and some highly repetitive polypeptides, including at least some viral capsid proteins. In contrast to TD antigens which usually contain only one copy of a determinant, the repeating epitopes of Type 2 T-independent antigens allow for multivalent binding to B cells through the hypervariable regions of surface Ig molecules. When a TI-2 antigen encounters a B cell expressing cognate cell surface receptors, the antigen binds to multiple B cell surface receptors but is not internalized. A TI-2 antigen remains, unprocessed, on the cell surface and stimulates the T-cell independent pathway directly, without direct T cell intervention.

By binding to the hypervariable regions of multiple surface Ig molecules, TI-2 antigens can stimulate an antigen-specific response. Although this surface receptor cross-linking provides the initial signal for B cell proliferation, additional signals are required for antibody secretion. Generally, these second signals are provided by extrinsically produced cytokines.

In addition to receptor-mediated binding to B cells, TI-2 antigens also bind to the cell surface of a highly specialized population of macrophages, physically segregated in the marginal zone of the spleen and subcapsular lymph node sinus. These specialized antigen presenting cells do not elicit T cell help, but rather present T-independent antigens directly to B-cells. Notably, this antigen is merely trapped on the cell surface without endosomal processing or association with Class II MHC.

Not only do most TI-2 antigen molecules (e.g. bacterial polysaccharides) remain intact on the surface of macrophage and B cells, but even if internalized, these antigens are not degraded by endosomal

proteases, nor do they efficiently bind to MHC molecules; consequently, they cannot themselves enter the highly productive Class II pathway and without additional stimuli, these antigens can only induce the immunologically inferior T cell independent antibody response.

Thus, the immune response of even immunocompetent normal individuals to polysaccharide or other TI-2 antigens is, in general, of low magnitude and low avidity. In addition, it entails minimal class switching and little, if any, memory response. These characteristics reflect the minimal involvement of T cell-derived help in promoting immunity to TI antigens. To date, the most effective way of generating an immune response to polysaccharide antigens has been to conjugate proteins containing T cell epitopes to the polysaccharides (i.e., conjugate vaccines). These constructs enhance the anti-polysaccharide response, presumably by stimulating internalization and subsequent T cell help. While these conjugate vaccines provide benefit, those in the art recognize the many limitations and disadvantages associated with their use.

For example, even where individuals are able to mount an immune response to these antigens, as for example after vaccination with a large inoculum of antigen, that response may require the coadministration of adjuvants. The most common adjuvants used in man are aluminum compounds (phosphate and hydroxide), such as alum. Alum, however, does not adjuvant all antigens (for reasons not entirely clear but perhaps due to a charge effect) and both alum and other experimental adjuvants may cause undesirable, and even life threatening, inflammatory responses.

In contrast to TI-2 antigens, TI-1 antigens provide both the first (proliferative) signal, and also a second signal that stimulates antibody secretion. The most commonly known TI-1 antigen, Lipopolysaccharide (LPS), is comprised of polysaccharide conjugated to lipid A. As with TI-2 antigens, the repeating epitopes of the polysaccharide cross-link the hypervariable regions of surface Ig molecules to provide the first signal. Lipid A, a known mitogen for B cells and macrophages, provides the second signal

necessary to stimulate antibody secretion. TI-1 antigens produce only antigen-specific responses *in vivo* in low doses. At higher doses, these antigens elicit non-protective, if not physiologically dangerous, polyclonal B cell activation. Because of the possibility of polyclonal activation, and due to the limited specificities of known TI-1 antigens, this class of antigens is commonly considered to lack clinical value. Consequently, vaccines against polysaccharides and other T-cell independent antigens are commonly based on TI-2 antigens which do not themselves provide signals to stimulate antibody secretion.

The identity and source of the signals which stimulate antibody secretion is currently under investigation. In related patent application Serial 08/315,492, filed September 30, 1994, incorporated herein by reference, the inventors previously demonstrated that B cells activated by anti-IgD antibodies conjugated to high molecular weight dextran ($\alpha\delta$ -dex), a construct that mimics the repetitive nature of the Type-2 T cell-independent antigens, required the presence of one or more of the cytokines IL-5, IL-3, GM-CSF, and/or IFN- γ to induce strong Ig secretory responses *in vitro*. Under these conditions, the optimal Ig-inducing activity of IL-3, GM-CSF, and IFN- γ , required costimulation with IL-2. The implication of this work is that TI-2 antigens *in vivo* may not be able to stimulate effective Ig responses in the absence of cytokines.

The physiologic source of the cytokines which are required for immune responses to TI-2 antigens is, at least partially, T cells. Although some literature suggests that T cells are not necessary to raise a response to polysaccharides, and that T cells merely modulate the TI-2 response, these studies have been based on the injection of large doses of highly purified (and sometimes also haptenated) polysaccharides which do not adequately reflect the physiological conditions of *in vivo* infection.

Thus, despite the historical designation "T cell-independent immunity," an effective TI-2 response *in vivo* is at least indirectly dependent on functional T cells. For example, studies by the Applicants demonstrate

that *in vitro*, sort-purified populations of B cells require T cells or T cell derived help to secrete immunoglobulin in response to TI-2 antigens. Mond et al., J. Immunol 131:633-37 (1983) (incorporated herein by reference). More recently, the Applicants have shown that administration of the poorly immunogenic *native* polysaccharide of whole bacteria to normal hosts results in both IgM and IgG antibodies, while in the absence of T cells only an IgM response is elicited. Snapper et al., unpublished.

Recently, it has been recognized that other cells, including NK cells, dendritic cells, and monocytes, also produce cytokines which may be important for the TI-2 response. Unfortunately, many immunocompromised patients, such as neonates, the elderly, those infected with HIV, individuals expressing genetic mutations affecting the immune system, or patients undergoing chemotherapy, may not have the functional complement of T cells, NK cells, dendritic cells, and/or monocytes necessary to produce adequate amounts of cytokines for optimal humoral immunity. Without additional help, these patients may not be able to mount an effective defense against TI-2 antigens.

The specific etiology underlying immune deficiency may vary from patient to patient. It has long been documented that the humoral antibody response declines with age. Consequently, infections become a major cause of death and ill-health among the elderly. As the decline in humoral immunity arises concomitant with the involution and atrophy of the thymus, it has been hypothesized that the decline in B cell response reflects a failure of T cell help, and restriction in the B cell repertoire.

Neonates are also generally deficient in generating humoral immune responses, with the greatest deficit observed in responses to TI-2 antigens relative to T cell-dependent or Type I T cell-independent antigens. The mechanisms underlying the reduced TI-2 response are complex, but appear to relate in large part to the intrinsic, functional immaturity of both neonatal T and B lymphocytes, including defects in membrane Ig-mediated signaling. Neonatal B cells respond poorly to a number of individual B cell

activators and various cytokines, relative to adult B cells. Further, when neonatal B cells are stimulated to secrete immunoglobulin, those antibodies are primarily IgM, a primitive antibody whose presence may suggest a general deficiency in Ig class switching.

The primitive IgM antibodies are initially secreted by B cells upon contact with a specific antigen. Subsequent genomic rearrangements in the secreting B cell result in isotype class switching, whereby the constant region of the IgM heavy chain is replaced with different constant regions, and thus generating IgA, IgE, or IgG antibodies. Class switching is a general indicator of maturation of the humoral immune response, occurring concomitantly with the appearance of higher affinity antibodies. Consequently, deficiencies in class switching which result in a predominantly IgM response will generally produce low avidity antibodies. In addition, IgM antibodies, in contrast to IgG, penetrate tissues only poorly and do not cross the placenta.

Deficiencies in immunoglobulin isotype class switching are also common among other classes of immunocompromised individuals. At the extreme are individuals affected by Hyper-IgM syndrome, characterized by increased levels of IgM associated with the virtual absence of IgG and IgA isotypes. The underlying genetic cause of Hyper-IgM syndrome in some patients is known to be a mutation in the gene encoding CD40 ligand. In other patients, some intrinsic deficiency of B cells prevents activation of the CD40 pathway. CD40 ligand is present on T cells and is normally required for T-cell mediated responses, including induction of the B cell memory response and immunoglobulin class switching. Unable to mount a complete and effective secondary immune response, patients afflicted with Hyper-IgM syndrome are susceptible to opportunistic infections.

Similarly, patients afflicted with DiGeorge and Nezelof syndromes lack a fully functioning thymus; consequently, the T cells in these patients fail to develop normally or may be entirely absent. As may be expected, the response to T-dependent antigens is minimal to non-existent and these patients rely predominantly on the T1-2 humoral response. Further, not only

do these patients lack a normal T-dependent response, but the paucity of T cells may even limit the effectiveness of the remaining TI-2 response.

In addition to genetic and developmental immunodeficiency, secondary defects in the humoral response are frequently the result of pathogenic infection. Immunosuppressive effects have been attributed to Newcastle disease (in birds), rinderpest (in cattle), lepromatous leprosy, malaria, and measles, and the various human immunodeficiency viruses.

The most notorious human immunodeficiency virus, HIV, results in the gradual depletion of T-4 helper lymphocytes, and the concomitant loss of T cell dependent immunity. Consequently, the remaining humoral response is primarily a T cell-independent one. Furthermore, the destruction of lymphocytes in these patients reduces the efficacy of the T cell independent response, most likely by reducing the availability of T cell-derived cytokines.

In addition to pathogenic affects, secondary defects in the humoral immune response can also be acquired by occupational, accidental, or medical exposure to radiation, cytotoxic chemicals, corticosteroids and other immunosuppressive drugs. Patients suffering from acute or chronic immunodeficiencies in response to these environmental factors present an increased risk of life-threatening infection.

Thus, there is a longstanding need in the art for methods of promoting class switching and memory responses to Type 2 T cell-independent antigens, most particularly in neonates, the elderly, and other immunocompromised patients.

SUMMARY OF THE INVENTION

The present invention addresses these needs by providing compositions for and methods of promoting a vigorous immune response to Type 2 T cell-independent antigens and TI-2-associated haptenic groups, by the administration of these antigens as conjugates with a lipid or fatty acid, or lipid-containing moiety such as lipoproteins, lipopeptides, or lipoamino acids. The lipid or lipid-containing moiety may be synthetic, semi-synthetic or derived from any prokaryotic or eukaryotic source including bacteria, fungi,

animals, and plants. In a preferred embodiment, the lipid or lipid-containing moiety is a synthetic or microbial lipoprotein. In a more preferred embodiment, the lipoprotein of the invention is Lipoprotein D (Lipo D). In a still more preferred embodiment, the lipoprotein of the invention is Lipoprotein OspA (Lipo OspA).

The present invention may be successfully used in normal adult patients and is particularly useful in stimulating an immune response in immunocompromised patients, such as neonates, the elderly, those infected with HIV and other immunosuppressive pathogens, individuals expressing deleterious genetic mutations affecting the immune system, and patients exposed to immunosuppressive agents, for example, chemotherapeutics and radiation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions for and methods of promoting a vigorous immune response to Type 2 T cell-independent antigens and TI-2-associated haptenic groups, by the administration of these antigens as conjugates with lipid or lipid-containing moieties such as microbial or synthetic lipoproteins. These conjugates exhibit the extraordinary property of promoting an immune response to TI-2 antigens. Preferably, the invention relates to the promotion of a vigorous response to TI-2 antigens, characterized by increased immunoglobulin isotype (class) switching and/or immunologic memory (anamnestic response).

In eliciting an immune response the conjugate may be co-administered and/or conjugated to additional moieties such as CD 40 ligand, T cell dependent antigens, and one or more cytokines such as IL-1, IL-2, IL-3, GM-CSF, IFN- γ , and more preferably, IL-4 and IL-5. One method of administering CD 40 ligand and/or cytokines would be by chemical or genetic fusion with a relevant protein or peptide wherein the resultant chemical or recombinant fusion protein has cytokine activity.

As used herein, the immune response is the body's production of immunoglobulins, or antibodies, in response to a foreign entity. Promoting or

stimulating an immune response refers to establishing an immune response that did not previously exist; to optimizing or increasing a desired immune response; or to establishing or increasing a secondary response characterized by increased isotype switching, memory response, or both. The measurement of the immune responses is within the ordinary skill of those in this art.

As used herein, a vigorous immune response to the conjugate of the invention refers to an immune response greater than that obtained by the unconjugated components in a comparable host. Although, a priori, there is no clear relationship between a level of antibodies and the level of protection that they will provide, when applied to compositions containing components derived from or directed against pathogenic organisms, a vigorous immune response comprises that sufficient to effect a statistically measurable immunoprotective effect as compared to unconjugated components in a comparable host.

The magnitude of a vigorous response may vary depending on factors such as the immune status of the host and the particular epitopes employed. However, as a point of reference, a vigorous secondary response to the conjugate in normal patients is clearly indicated by a total antibody titer of approximately 7 times that of the elicited by the unconjugated components. Alternatively, in terms of isotype switching, a vigorous secondary response in normal hosts is clearly indicated by an approximately 4-fold decrease in the ratio of IgM to IgG isotypes as compared to unconjugated components. Of course, the magnitude of a vigorous immune response is not limited to these numbers, and those in the art will recognize smaller increases as within the scope of a vigorous response.

As used herein, the memory response is the acquired characteristic of B cells whereby a second (or subsequent) encounter with a specific antigen elicits a greater production of antibody than did the primary exposure. The memory response may be associated with a faster immunological response, higher affinity antibodies, and/or immunoglobulin class switching.

Class switching refers to the process by which a B-cell changes the class of antibody secreted without appreciably changing its antigen specificity. As used herein, class switching refers to the change from the IgD or IgM isotypes to IgG, IgE, and/or IgA isotypes.

The TI-2 antigens of the invention make up one of the two known classes of TI antigens. TI-2 antigens are characterized by their linear nature and spaced highly repetitive determinants. Polysaccharide antigens are commonly classified as TI-2 antigens. These antigens share the general characteristics of large molecular weight, repeating antigenic epitopes, ability to activate the complement cascade, poor *in vivo* degradability and the inability to stimulate major histocompatibility complex (MHC) class II-dependent T cell help.

Among the medically relevant TI-2 antigens are those derived from the pathogens *Bordetella pertussis*, *Borellia burgdorferi*, *Campylobacter*, *Candida albicans*, *Escherichia coli*, *Shigella*, *Haemophilus influenzae*, *Neisseriae meningitidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Cryptococcus neoformans*, *Escherichia coli*, *Klebsiella*, *Yersina*, *Vibrio cholerae*, as well as viruses whose capsids contain highly repetitive multi-epitope polypeptides. Preferred TI-2 antigens include polysaccharides of *Haemophilus influenzae* type b polyribosyl-ribitol-phosphate (PRP), *S. Pneumonia*, Group B *Streptococcus*, *N. meningitides*, *Salmonella*, *P. aeruginosa*, and *P. aeruginosa* (including strain Fisher type 1) polysaccharides.

TI-2 antigens bind to antigen-specific B cells by cross-linking IgD or IgM receptors on the surface of the B cell, a process known as membrane (m)Ig-mediated signaling. (m)Ig-mediated signaling provides the stimulus for the B cell to proliferate. However, the TI-2 antigen alone cannot provide the necessary second signal that instructs the stimulated B cell to secrete antibody. This second signal is normally provided by cytokines produced by non-B cell types.

Type 1 T-independent antigens, such as lipopolysaccharide (LPS), also cross-link cell-surface IgD or IgM to provide the initial proliferative signal. In addition, TI-1 antigens contain a second B-cell activator. This moiety generates a second (secretory) signal which by-passes the cytokine requirement of TI-2 antigens. In the classic TI-1 antigen, LPS, the second B-cell activator is lipid A.

The other antigens of interest are known as T cell dependent (TD) antigens. They consist of soluble peptides or proteins, distinguishable from TI antigens by their ability to associate with Class II MHC molecules at the surface of an antigen presenting cell (APC). This association allows the antigen presenting cell to interact with T cells through the T cell antigen receptor complex (TCR), as well as through binding of accessory and adhesion molecules.

The TD antigens may be present in the invention in one of two ways. First, they may make up part of a lipid-containing moiety such as lipopeptide or lipoprotein, as detailed below. Thus, for example, two of the preferred lipid-containing moieties, Lipoprotein D (Lipo D) and Lipoprotein OspA (Lipo OspA), comprise T cell-dependent protein antigens covalently bound to lipid elements. As a consequence, the immune response to Lipo D and Lipo OspA conjugates includes antibodies directed not only to the TI-2 component but to the TD component of the lipoprotein as well.

Additional TD antigens may be present in another embodiment in which additional TD antigens of interest are included in the composition, preferably directly or indirectly conjugated to the TI-2 component. These TD antigens may be derived from a pathogenic organism and conjugated to the TI-2 component. Consequently, an immune response, or in a preferable embodiment, a vigorous immune response to TD antigens of pathogenic organisms may be elicited, even in a T cell deficient host.

In addition to the TI and TD antigens, haptens comprise a third class of potential antigenic moiety among the antigens of interest. Haptens refer to small molecules, such as chemicals, dust, and allergens, that by

themselves are poorly antigenic, or are incapable of eliciting an antibody response, unless coupled to a carrier. These haptens may derive from but are not limited to bacteria, rickettsiae, fungi, viruses, parasites, drugs, or chemicals. They may include, for example, small molecules such as peptides, di- and oligosaccharides (for example, small fragments of *H. influenzae* polyribosyl-ribitol-phosphate), toxins, endotoxin, and chemicals, (for example, m-aminobenzenesulfonates, trinitrophenol, and glycosides) etc. In the present invention an immune response to one or more hapten moieties may be elicited by directly or indirectly coupling the hapten to any TI-2, lipid or lipid-containing moiety, or non-lipid-containing TD component of a conjugate.

The ability of present invention to promote an immune response, including a vigorous immune response, to haptens, TD and TI-2 antigens may currently be best understood in light of recent research into the B cell response to TI-2 antigens. Although the inventors do not wish to be limited by the proposed mechanisms of action described below, much has been learned about (m)Ig-mediated signaling in response to TI-2 antigens based on a polyclonal *in vitro* model developed by the inventors. As fully explained in the parent application 08/568,342, and in U.S. applications 08/568,343, and 08/468,475, incorporated herein by reference, the inventors synthesized high molecular weight dextran-conjugated anti-IgD or anti-IgM antibodies (anti-Ig-dextrans) in order to simulate the repeating epitope nature of polysaccharides to create a multivalent antigen on a polysaccharide carrier. This procedure converts the bivalent anti-Ig molecule into an extremely stimulatory multivalent conjugate which can induce persistent and repetitive signaling via B cell membrane Ig, even at picomolar concentrations. The activation of B cells occurs irrespective of antigen specificity of the membrane bound Ig. However, α D-dex does not stimulate the release of antibody by small resting (unactivated) B cells in the absence of cytokines. With the addition of cytokines, high levels of Ig secretion and Ig class switching are observed.

In vivo, this requirement for cytokines severely hampers the treatment of immunocompromised patients. Immunocompromised patients, lacking a functional complement of cytokine-producing T and/or non-T cells, may fail to produce sufficient quantities of cytokines to support an effective TI-2 response, and thus risk infection from common pathogens including bacteria, fungi and viruses. Applicants have discovered that this deficiency can be alleviated by vaccinating immunocompromised patients with the Type 2 T cell-independent-lipid or lipid-containing conjugates (TI-2-L) of this invention which incorporate medically relevant TI-2 antigens such as those described above.

Among the lipid and lipid-containing moieties of the invention, lipoproteins have been previously shown to deliver non-(m)Ig-mediated signals to B cells. Melchers, et al., 49 J. Exp. Med. (1975) 142:473. Early studies on the B cell activating properties of lipoproteins, however, employed heterogenous populations of lymphoid cells in various stages of *in vivo*-preactivation and cultured at relatively high cell densities which tend to facilitate interactions of B cells with other cell types. Because these cells were not fractionated according to density and, hence, prior activation state, these studies left unresolved whether lipoproteins acted directly at the level of the resting B cell, whether additional cell types played key roles in their action, and how lipoprotein-mediated signaling integrated functionally with other B cell stimuli, including (m)Ig-mediated TI-2-like stimuli also present in bacterial cell walls.

In contrast to early studies, parent application 08/568,342 discloses that, in a highly-enriched and sort-purified population of resting B cells, lipoproteins must act in concert with other stimuli to induce strong proliferative and Ig secretory responses. When B cells are thus purified to remove pre-activated B cells and contaminating cell types, neither Lipo D, Lipo OSPA, nor synthetic lipopeptides such as Pam₃Cys derivatives by themselves stimulate significant proliferation or Ig secretion. However, in an *in vitro* model of B cell activation, these lipoproteins synergise with TI-2-like multivalent antigen

receptor cross-linking, to stimulate marked B cell proliferation and Ig secretion in the absence of exogenous cytokines. Moreover, this combination acts directly at the level of the B cell without a requirement for recruitment of non-B cell types *in vitro*. These data suggested an additional, novel pathway for induction of specific, T cell-independent humoral immunity which may be particularly applicable to bacterial antigens. Thus, in view of the dramatic *in vitro* response, the conjugates of the invention were developed comprising a polysaccharide antigen conjugated to a lipid or lipid-containing moiety with the view of eliciting a primary immune response preferably a strong primary response, to the polysaccharide.

The conjugates of the present invention elicit a greater primary immune response than do the unconjugated components. In a preferred embodiment, this enhanced response is typically approximately 10-fold greater than the response to unconjugated components in a immunocompetent host, and has been shown to be approximately 8-fold greater in an immunocompromised host, although the response produced by the invention is not limited to these values. The synergistic effect of combining the (m)Ig-mediated proliferative signal of TI-2 antigens, with the secretory signal of lipids or lipid-containing molecules is not predicted or suggested by the prior art. Indeed, there is no biochemical or other basis for anticipating that these signals acting in concert would have more than additive effects.

Surprisingly, the compositions of the invention also exhibit the unexpected ability to stimulate an antigen-specific secondary response, preferably a vigorous secondary response, to the TI-2 component. As set forth herein, this response is characterized by extensive isotype class switching and memory response to antigens traditionally considered refractory to strong secondary responses. More surprisingly, this secondary response is not T cell dependent and occurs in hosts completely lacking T cells.

Moreover, although NK cells have been suspected to be involved in the TI-2 response, the complete ablation of both T cells and NK cells greatly reduces, but does not entirely eliminate, the humoral immune responses of the present invention. Further, in the complete absence of both cell types *in vivo*, the response to the basic TI-2 - lipid or lipid-containing (TI-2-L) conjugate continues to exceed the response to the unconjugated components in similar animals.

Although the immune response to the conjugates of the invention is reduced in the absence of both T and NK cells, this effect will be ameliorated by further conjugating and/or co-administering one or more immunostimulatory moieties such as CD 40 ligand or more cytokines such as IL-3, GM-CSF, IFN- γ , and more preferably, IL-4 and IL-5. These additional effector molecules will thereby replace, or substitute for, those factors formerly supplied by the T and NK cells. In addition, the further conjugation and/or co-administration of one or more of the above effector molecules will increase the immune response to the TI-2-L construct in hosts that do not lack both T and NK cells.

Of the above effector molecules, the CD40 ligand is normally present on T cells and has generally been considered essential for T-cell mediated responses, such as induction of B cell memory and immunoglobulin class switching. However, the conjugates of the invention induce even a vigorous immune response to TI-2 antigens in the complete absence of T cells, demonstrating a response that is independent of the presence of CD40 ligand. Thus, this response defines a novel non-CD40 ligand dependent pathway. The conjugates of the invention are thus useful in promoting an immune response, and preferably a vigorous immune response in patients exhibiting defects in the CD40 ligand pathway, most obviously, those afflicted with Hyper-IgM syndrome.

Further, patients deficient in T cells generally also lack CD40 ligand, and thus lack functional CD40 ligand-dependent responses. With the addition of CD40 ligand, the conjugates of the invention may thus be used to

promote isotype switching and B cell memory response to TD as well as TI-2 antigens in the absence of T cell help. Consequently, with the addition of CD40 ligand the conjugates of the invention may be particularly useful in augmenting a poor immune response to TD antigens. Indeed, previous work by the Applicants using highly purified B cells demonstrates that Lipoprotein D synergises with CD40 ligand to stimulate DNA synthesis and Ig secretion. Snapper et al., J. Immunol. 155:5582-89 (1995), incorporated herein by reference.

In addition to their role in laboratory investigations of the immune system, the TI-2-L conjugates of the invention provide a valuable clinical tool. Neonates and infants, for instance, exhibit markedly defective TI-2 responses with increased susceptibility to infections from polysaccharide-encapsulated bacteria. *In vitro*, neonatal B cells respond poorly to a number of individual B cell activators and various cytokines, compared to adult B cells. The inventors have recently discovered that costimulation of highly purified $\alpha\delta$ -dex stimulated neonatal B cells with any one of CD40 ligand, LPS, or Lipo OspA, dramatically increases Ig secretion *in vitro*. Upon the further addition of IL-4 and IL-5 to the culture media, the IgM secretory response of neonatal cells is further enhanced to close to or above adult levels. (Snapper et al., J. Immunol. 158:2731-35 (1997) (incorporated herein by reference). These results demonstrate that neonatal B cells are competent to secrete Ig *in vitro* if adequate costimuli are provided. The TI-2-L conjugates of the invention will provide those stimuli.

The lipids and lipid-containing moieties of the present invention may be from any source, including plant and microbial origin, wholly or partially synthetic, or recombinant. The microbial-derived lipid-containing moieties are generally components of bacterial cell walls and include, but are not limited to, the distinct lipoproteins that have been identified in the cell walls of different bacteria. Erdile et al., Inf. and Imm. (1993) 61:81. For use in the current invention, these lipoproteins may also be derived from the genes encoding them, such as Lipoprotein-Osp B from *Borrelia burgdorferi*

(see Ma et al., J. Infect. Dis. 171:909-15 (1995), lipoprotein-D from *Haemophilus influenzae* and, more preferably, Lipoprotein-OspA from *Borrelia burgdorferi*. Id. and see Song et al., Infect. & Immun. (1995) 63(2):696. The lipoproteins and other lipid-containing moieties of the claimed invention also include fragments, or sections thereof, that impart the proliferation and Ig secretion actions observed with Lipoprotein D or Lipo OspA. These fragments may be derived by any technique known in the art including enzymatic digestion of full-length protein, chemical synthesis, or the generation of molecularly engineered expression products, including fusion proteins.

The lipids, lipoproteins and other lipid-containing moieties of the present invention also include synthetic lipid moieties which are structurally similar to the amino termini of bacterial lipoproteins. When these synthetic lipid moieties are conjugated to a small peptide or amino-acid, they can mimic the B cell-activating properties of these molecules. Further, removal of this lipid moiety from bacterial lipoproteins renders them non-functional. Some of the synthetic lipid-containing moieties of the invention well known in the art and are typified by the Pam₃Cys lipopeptide family (Klein, B., et al., Immunology 61:29 (1987); Reiterman et al., Biol. Chem. Hoppe-Seyler 370:343-52 (1989); and Jung et al., Angew. Chem. Int. Ed. Engl. 24:872-73 (1985)), which are all incorporated herein by reference. The synthetic lipid-containing moieties of the invention further include those based on N-palmitoyl-S-(2,3-bis(palmitoyloxy))-(2RS)-propyl)-(R)-cysteinyl-(S)-seryl-(S)-asparaginyl-(S)-alanine (Bessler and Young, Res. Immunol. 143(5) 471-586 (1992)) and tripalmitoyl-S-glyceryl-cysteinylserylserine (Wiesmuller et al., Vaccine, 7:29-33 (1989)), which are also incorporated herein by reference.

The lipids and lipid-containing moieties of the present invention are physically or chemically bound to the TI-2 antigen, directly, through a linker, or through another moiety such as a hapten, protein, carbohydrate or allergen. In the preferred embodiment, the lipids or lipid-containing moiety is directly conjugated to the antigen. Additional TD antigens (exclusive of any

comprising the lipoprotein) and haptens may be physically or chemically bound to the TI-2 antigen, or to the lipid or lipid-containing moiety, either directly, through a linker, or through another moiety. In a preferred embodiment, additional TD antigens are directly bound to the TI-2 antigen. In a preferred embodiment, haptens are directly bound to the TI-2 antigen, to a TD antigen, or both.

Any form of chemical binding, including covalent, is within the scope of this invention. Methods of conjugation are well known to those of ordinary skill in the art, and include the heterologation techniques of Brunswick et al., J. Immunol., **140**:3364 (1988); Wong, S.S., Chemistry of Protein Conjugates and Crosslinking, CRC Press, Boston (1991); Brenkeley et al., "Brief Survey of Methods for Preparing Protein Conjugates With Dyes, Haptens and Cross-Linking Agents," Bioconjugate Chemistry, **3**, No. 1 (Jan. 1992); and Hermanson, G.T. Bioconjugate Techniques, Academic Press, San Diego (1996), each of which are specifically incorporated by reference.

A preferred method of covalent conjugation is via CDAP (1-cyano-4-"dimethylamino"-pyridinium tetrafluoroborate) activation of the polysaccharide, set forth in applications Serial No. 08/482,616, and 08/482,666, filed June 7, 1995, (08/482,616 being now abandoned); which are a continuation-in-part applications of application Serial No. 08/408,717, filed March 22, 1995, and issued July 29, 1997, as U.S. Patent No. 5,651,971, and which is a continuation-in-part of application 08/124,491, filed September 22, 1993, now abandoned, and further set forth in the continuation of application 08/408,717, application 08/456,694, filed June 1, 1995, which issued December 2, 1997 as U.S. Patent No. 5,693,326, and as further set forth in in the continuation-in-part of application 08/124,491, application 08/124,491, filed September 22, 1993, the disclosures of which are all specifically incorporated herein by reference.

Treatment comprises administering the pharmaceutical composition by any method familiar to those of ordinary skill in the art, including intravenous, intraperitoneal, intracorporeal injection, intra-articular,

intraventricular, Intrathecal, intramuscular, subcutaneous, topically, intranasally, intravaginally, orally. The preferred methods of administration are intravenous, intramuscular, intranasal, oral, and subcutaneous injections. The composition may also be given locally, such as by injection into the particular area, either intramuscularly or subcutaneously.

The compositions of the invention may be considered pharmaceutical compositions in that they elicit a biological effect on the immune system. When the pharmaceutical composition of the invention is to be administered to an organism, preferably suspended or dissolved in a pharmaceutically acceptable carrier, it may be referred to as a vaccine.

The conjugate may also be expressed in transgenic plants or plant products. The vaccine may then be administered orally as part of the plant or plant product. Alternatively, the conjugate may be purified from the plant or plant product prior to administration. When the conjugate is expressed in plants it is preferred that the TI-2 antigen comprise a highly repetitive polypeptide sequence such as that identified in some viral capsid proteins.

The compositions of the claimed invention may be applied to isolated B cells *in vitro* as a pharmaceutical composition or administered directly to the patient as a vaccine. Such pharmaceutical compositions and vaccines specifically include compositions comprising lipoprotein physically or chemically conjugated to the dual conjugate vaccines of application Serial No. 08/468,060, filed June 6, 1995 (a continuation-in-part of application Serial No. 08/402,565, filed March 13, 1995, which issued on December 17, 1996, as U.S. Patent No. 5,585,100) and the dual conjugate compositions of application Serial No. 08/444,727, filed May 19, 1995 (a continuation of 08/055,163, filed February 10, 1993), the disclosures of which are specifically incorporated herein by reference. The pharmaceutical compositions and vaccines of the invention may also include at least one lipid or lipid containing moiety physically or chemically conjugated to the polysaccharide-TD antigen compositions described in Dick and Beurret, Conjugate Vaccines, in Contrib. Microbiol. Immunol. (1989), incorporated herein by reference.

A patient is hereby defined as any person or non-human animal in need of immune stimulation. Such non-human animals to be treated include all domesticated and feral vertebrates, preferably, but are not limited to mice, rats, rabbits, dogs, cats, swine, horses, cattle, fowl, and non-human primates. In the preferred embodiment the patient is a neonate, aged, infected with HIV, or otherwise immunocompromised or T cell depleted.

Secondary booster immunizations may be given at intervals ranging from one week to many months later. The dosage of the primary and secondary inocula can be readily determined by those of ordinary skill in the art, but an acceptable range is 0.01 μ g to 100 μ g per inoculum.

As used herein, a vaccine comprises at least one TI-2-L conjugate, preferably dissolved or suspended in a pharmaceutically acceptable carrier or vehicle. Any pharmaceutically acceptable carrier can be employed for administration of the composition. Carriers can be sterile liquids, such as water, oils, including petroleum oil, animal oil, vegetable oil, peanut oil, soybean oil, mineral oil, sesame oil, and the like. With intravenous administration, water is a preferred carrier. Saline solutions, aqueous dextrose, and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Remington's Pharmaceutical Sciences, 18th Edition (A. Gennaro, ed., Mack Pub., Easton, Pa., 1990), incorporated by reference.

A further object of this invention is to provide a novel assay system for identifying compositions useful for stimulating isotype switching and/or the B cell memory response. This assay comprises administering a TI-2-L conjugate to B cells, and then measuring the amount of isotype switching and/or the B cell memory response. Using this assay, one of ordinary skill in the art can determine the precise composition of conjugate, cytokines, haptens, TD antigens, and other moieties most useful for eliciting a particularly secondary immune response. This assay is applicable both *in vivo*, preferably following genetic (e.g. T-cell knockout mice) or biochemical ablation of T cells (described in Example 2), and *in vitro*, using purified B

cells. *In vitro*, the B cells are preferably sort-purified to 98%, or still more preferably, 99% or more pure B cells, as described in U.S. application 08/315,492, incorporated herein by reference.

The invention will be further clarified by the following examples, which are intended to be purely exemplary of the invention.

Example 1

Materials and Methods

Female DBA/2 and nude mice were obtained from the National Cancer Institute (Frederick, MD). C57 BL/6 T-cell knockout (TCR β KO), CBA.B6 F₁, and CBA.B6 F₁ CD3 ξ transgenic mice were obtained from Jackson Labs (Bar Harbor, ME). All mice were used at 7-10 weeks of age. The experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Animal Resources, National Research Council, Department of Health, Education, and Welfare Publ No. (National Institutes of Health) 78-23.

Hib Polysaccharide, polyribosyl-ribitolphosphate, and *S. pneumoniae* polysaccharide 6B and 14, obtained from ATCC, were extracted and purified from inactivated cell cultures, using standard methods. The purified material met the WHO and US specifications in terms of residual protein, nucleic acid, endotoxin, structural sugars and molecular size distribution. *Haemophilus influenzae* lipoprotein D was expressed in *E. coli* and purified using conventional column chromatography. The purity of the proteins was above 90% as assessed by different methods (SDS-PAGE, CE, HPLC). The activated Hib PS, *S. pneumoniae* polysaccharide 6B or *S. Pneumoniae* polysaccharide 14 were conjugated to lipoprotein D or tetanus toxoid, as set forth below.

Direct conjugation of a polysaccharide and a lipoprotein using CDAP (1-cyano-4-"dimethylamino"-pyridinium tetrafluoroborate) is essentially as described in Lees, WO 95/08348, incorporated herein by reference. Specifically, Pn14 polysaccharide is suspended in saline @ 5 mg/ml on ice, CDAP @ 100 mg/ml in acetonitrile, 0.2 M TEA (triethylamine), 6 mg/ml

lipoprotein D in 10 mM sodium phosphate, 0.2 M NaCl, 0.1% Empigen (a detergent from CalBiochem) pH 7.2, on ice. Activation and coupling are performed at 0-4°C. CDAP is slowly added to a stirred solution of Pn14 at a ratio of 0.75 mg CDAP/mg Pn14. At 30 seconds, the pH is raised to 10 with TEA (usually about 2x the volume of CDAP used) and maintained at pH 10 for a total of 2 minutes with TEA. After a total of 2.5 minutes, the lipoprotein D is added to the activated polysaccharide, while mixing, at ratio of 2.5 mg protein/mg Pn14. The pH should be in the range of 9-9.5. After one hour, the reaction is quenched by the addition of one quarter volume of 1 M glycine @ pH 8. After an overnight incubation at 4° C, the conjugate is purified by passage over an S500HR (Pharmacia) gel filtration column. The high molecular weight conjugate, containing protein and polysaccharide, is pooled and filtered through a 0.2 micron filter. Protein is determined using the Lowry assay, polysaccharide using a resorcinol assay.

Direct conjugation of a polysaccharide and a lipoprotein using CDAP Coupling via a spacer may be affected as follows: Pn14 is activated with CDAP as above. At 2.5 minutes, one half volume of 0.5 M adipic dihydrazide at pH 8 is added. After one hour, the solution is exhaustively dialyzed into saline. Hydrazide content is measured using TNBS, polysaccharide using a resorcinol assay. Lipoprotein may then be coupled to the Pn14-hydrazide as described by Lees, et al., Vaccine, 1994, 12, 1160, incorporated herein by reference. In brief, the Pn14-Hydrazide is iodoacetylated with iodoacetyl N-hydroxysuccinimide (SIA, Sigma). The protein is thiolated with S-Acetylthioacetyl N-hydroxysuccinimide (SATA, Sigma). Following desalting and concentration using a Centricon 30 device, the two are combined and the pH raised to 7.5 using 1/9 volume of 0.75 M HEPES, 0.5 M hydroxylamine. After an overnight reaction at 4° C, the reaction is quenched with mercaptoethanol (Aldrich) @ 0.2 mM for one hour, followed by iodoacetamide (Aldrich) @ 10 mM for 10 minutes. The conjugate is purified as described above.

Anti-Lipo D antibodies were measured by ELISA using protein D as coating antigen. Anti-Hib PS antibodies were measured by ELISA using tyraminated Hib PS for coating and, for anti-PS 6B or 14, absorption of anti-CPS antibodies was performed by addition of CPS (coupled polysaccharide) to the serum (1 mg/ml for anti-PS 14 and 5 mg/ml for anti-PS 6B). The detection of specific antibodies was made using an anti-rat conjugate labeled with peroxidase. For all the ELISA's, a reference serum was used and the titers were calculated in arbitrary units using the 4-parameter methods.

Immunoglobulin isotype concentrations were measured by an ELISA assay. For determination of concentrations of secreted IgM, IgG3, (IgG1, IgG2b, IgG2a), and IgA in culture SN, Ameslan 2, 96-well flat-bottomed ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with unlabeled affinity-purified polyclonal goat anti-mouse IgM, IgG3, IgG, and IgA antibodies (Southern Biotechnology Associates, Birmingham, AL), respectively. Plates were then washed, blocked with fetal bovine serum (FBS)-containing buffer, and incubated with various dilutions of culture SN and standards. After washing, plates were incubated with alkaline phosphatase-conjugated affinity-purified, polyclonal goat anti-mouse IgM, IgG3, IgG1, IgG2b, IgG2a, and IgA antibodies (Southern Biotechnology Associates) as indicated, washed again, and a fluorescent product was generated by cleavage of exogenous 4-methyl umbelliferyl phosphate (Sigma) by the plate-bound alkaline phosphatase-conjugated antibodies. Fluorescence was quantitated on a 3M FluoroFAST 96 fluorometer (Mountainview, CA) and fluorescence units were converted to Ig concentrations by interpolation from standard curves that were determined with known concentrations of purified myeloma Ig. Each assay system showed no significant cross-reactivity or interference from other Ig isotypes (IgM, IgD, IgG3, IgG1, IgG2b, IgG2a, and IgA) found in the culture supernatants.

Example 2

Lipoprotein D can enhance the primary anti-polysaccharide response in T-cell deficient animals

Mice were injected with 500 µg - 1.0 mg of an anti-CD4 antibody (GK1.5, ATCC number TIB 207) to induce T cell depletion, basically as described in Finkelman et al., Polyclonal Activation of the Murine Immune System by a Goat Antibody to Mouse IgD. IX. Induction of a Polyclonal IgE Response. J. Immunol. 138:2826-2830 (1987), incorporated herein by reference. One day later, the mice were injected with 5.0 µg of either pneumococcal polysaccharide type 14-lipoprotein D ("PN14-LPD") or PN14 alone. Fourteen days later, IgG1 anti-PN14 responses were measured by ELISA assay. As set forth below in Table 1, only the lipoprotein D conjugate stimulated high levels of anti-polysaccharide response in T cell depleted mice.

Table 1

TI-2-L conjugate vaccine with lipoprotein D as a component stimulates a vigorous primary anti-polysaccharide response in T cell depleted mice

Antigen	IgG, anti-PN14 titer (ng/ml)
PN14	< 10
PN14-Lipo D conjugate	5,834

Accordingly, the conjugates of the invention may be a valuable tool to enhance anti-polysaccharide, and other TI-2 antigen responses, in T cell deficient individuals, such as those undergoing chemotherapy or suffering from AIDS.

Example 3

The compositions of the invention elicit a vigorous secondary response to both the TI-2 antigen and T-dependent component in immunocompetent hosts

Immunogenic responses to bacterial polysaccharide antigens are commonly elicited by coupling the poorly immunogenic TI-2 antigen to a potent T dependent antigen such as tetanus toxoid (TT). To compare the

efficacy of tetanus toxoid and lipoprotein D on the TI-2 response, different vaccines based on the polysaccharide *Haemophilus influenzae* type b polyribosyl-ribitol-phosphate (PS) were prepared. These "Hib vaccines" included either tetanus toxoid or lipoprotein D as the source of T cell epitopes coupled to the polysaccharide by CDAP activation.

Groups of 10 female 5 week old OFA rats were immunized twice subcutaneously 4 weeks apart with 1/4 of a H.D. (high dose tolerance) of the vaccines, and bleeds were taken on day 28, 42, 56, 69 and 83. Anti-PS response evaluated by ELISA (coating with tyraminated-PS obtained by CDAP conjugation of tyramine and polysaccharide essentially as described in Example 1, above). A non-parametric method called "Robust" was used for the comparison of the anti-PS titers induced by different preparations. HIB 001A44 served as reference.

The anti-polysaccharide response to the various conjugates is reported in Table 2. The lipoprotein D TI-2-L conjugate, PS-Lipo D, induced a primary anti-polysaccharide response comparable to that of the tetanus toxoid conjugate, but induced a much higher (> 10X) anti-polysaccharide response following a boost injection than did the tetanus toxoid conjugate. Thus, the TI-2-L conjugates of the invention may be preferable to commonly used non-lipidated conjugates in inducing vigorous secondary-responses to polysaccharide antigens in immunocompetent hosts. To assess the effect of combined vaccines, a DTPa.HB vaccine (Diphtheria, tetanus toxoid, acellular pertussis with Hepatitis B (batch 16710)) was combined with the conjugates. The combination did not diminish the anti-polysaccharide response.

Table 2
Anti-PRP responses in rats immunized with Hib vaccines
combined or not with a DTPaHB Vaccine

Conjugate		Anti-PS response (ng/ml) to:			
Type (batch no.)	PS/Prot ratio	Conjugate Alone		Conjugate - DTPa HB	
		D 28	D 42	D 56	D 69
Solvent		0.05	0.11	0.15	0.17
<u>CDAP Activation</u>					
PS-TT (C294)	1/1	0.3	5	4.1	4.7
PS-TT (C295)	1/2	0.6	18	16	7.8
PS-Lipo D (001)	1/1	1.0	222	201	54

As set forth in Table 3, the PS-Lipo D conjugate induced primary and secondary anti-Lipo D titers comparable to the anti-protein titers induced by the tetanus toxoid conjugates. Anti-TT (values between brackets) titers were determined in sera of saline-injected rats.

Table 3
Anti-protein responses in rats after immunization with Hib vaccines

Conjugate		Anti-protein response (anti-TT or anti-LipoD titer)			
Type (batch no.)	Ps/Prot	D 28	D 42	D 56	D 69
Solvent		0.03 [1.8] [2]	0.02 [2.3]	0.01 [3.8]	0.01 [3]
<u>CDAP Activation</u>					
PS-TT (C294)	1/1	0.25	1.4	1.6	2.0
PS-TT (C295)	1/2	0.80	17.2	9.5	8.4
PS-Lipo D (001)	1/1	1.6	8.4	10.9	14

Example 4

Conjugated TI-2 - L compositions promote a vigorous secondary response in normal, immunocompromised, and T cell deficient patients

Cohorts of 5 mice representing normal (DBA/2), immunocompromised (nude), and T cell deficient (TCR-KO) patients, were immunized subcutaneously on day 0 with 2.5 µg each of either polysaccharide Pn14 conjugated to Lipo OspA, Pn14 conjugated to Lipo-D, or an unconjugated mixture of Pn14 and Lipo OspA. Identical boost injections were administered on day 14. Blood samples were taken on day 14 and day 28 and analyzed as described above for anti-Pn14 antibodies of various isotypes. The results are presented in Table 4.

Table 4

Conjugated TI-2-L compositions promote a class switching and memory response in normal and T cell deficient mice

Strain	Antigen	bleed	Anti-PN14 titer (ng/ml)			
			IgM	IgG ₁	IgG _{2a}	IgG ₃
DBA/2 (Normal)	Pn14-Lipo OspA conjugate	1°	4287	16986	3262	2307
		2°	1430	131766	38465	22386
	Pn14-Lipo-D conjugate	1°	1151	4908	920	943
		2°	2110	100599	24049	19713
	Pn14+ Lipo OspA unconjugated	1°	456	187	167	117
		2°	438	139	78	52
nude (Immuno- compromise d)	Pn14-Lipo OspA conjugate	1°	1846	2283	2092	1071
		2°	2105	6436	2976	3802
	Pn14-Lipo-D conjugate	1°	1230	865	701	259
		2°	1524	15901	4168	6373
	Pn14+Lipo OspA unconjugated	1°	540	127	114	93
		2°	740	292	230	132
TCR-KO (T-cell deficient)	Pn14-Lipo OspA conjugate	1°	438	184	154	54
		2°	1295	93677	9369	17694
	Pn14-LPD conjugate	1°	630	137	97	51
		2°	727	12890	3036	2946

Administration of a composition comprising the TI-2 antigen, Pn14, and either Lipo OspA or Lipo D to immunocompetent DBA/2 mice promotes a vigorous anti polysaccharide response only when the polysaccharide is physically conjugated to the lipid-containing moiety. If the TI-2 antigen and the lipoprotein are not physically linked, the overall immune response is comparatively attenuated, and further reflects the common property of TI-2 responses of being heavily weighted toward IgM isotype antibodies. Unlinked components elicit 49% and 62% IgM following primary and secondary injections as compared to more mature IgG isotypes. In contrast, the same components, chemically coupled to form an embodiment of the invention promote significant class switching among the anti-polysaccharide antibodies, even when assayed as early as 14 days after the primary exposure. Where the polysaccharide is conjugated to Lipo-D, the isotype ratio is only 14% IgM measured after either injection. Even more dramatically, the isotype ratio elicited in response to the polysaccharide - Lipo OspA conjugate drops to a mere 0.7% IgM following a booster injection. These low IgM/ IgG ratios are indicative of a highly mature secondary response.

In dramatic contrast to the unconjugated antigens, a second injection of conjugated antigen promotes a 7 fold increase in anti-Pn14 antibodies where the polysaccharide is conjugated to Lipo OspA and an 18 fold increase where the lipoprotein is Lipo D. The large increase in humoral response following a second injection provides a clear indications of a vigorous memory response to the TI-2 antigen.

As previously indicated, TI-2 antigens respond poorly, if at all to boost injections, such that initial and subsequent exposures to the antigen generate the characteristically poor primary immune response, heavily weighted to the IgM isotype. In contrast, conjugates of the invention elicit a high titer following primary injection, substantial class switching, and clear evidence of memory response to TI-2 antigens.

One object of the invention is to generate a vigorous immune response in immunodeficient patients. The congenitally thymus deficient

nude mouse has long been recognized as a model for some immunodeficiencies. Because the T cells of nude mice necessarily develop in an extrathymic pathway, the mice are highly deficient in T cells. Moreover, the remaining population of T cells does not reflect the normal ratio of TCR sub-types. T cells may be defined by the pair of T cell receptor (TCR) molecules expressed on the cell surface, where each T cell expresses either α and β , or γ and δ polypeptides. In a normal individual, TCR $\alpha\beta$ cells predominate, whereas in nude mice, the majority of T cells are TCR $\gamma\delta$.

Lacking a functional TCR complement, nude mice cannot mount a classical T dependent response and cannot respond to soluble proteins in adjuvant. However, the response to T independent antigens is relatively normal in these animals. Thus, although nude mice represent a highly artificial model of immunodeficiency, they have long been used to dissect T cell independent immune responses, and as a probe to identify immunologic protocols which may depend on T cells.

The results in Table 4 demonstrate that use of the composition does not require an intact T cell complement. Even in nude mice, the conjugated compositions of the invention promote robust primary and secondary immune responses. The anamnestic response is particularly striking for the Lipo OspA conjugate, wherein the anti-Pn14 titer increases to 11 fold in response to the boost injection. Class switching is also apparent, even following the primary injection, and increases substantially after the boosting vaccination.

These experiments demonstrate that the immunological response to the conjugates of the invention does not depend on an intact T cell repertoire. Thus the invention is useful for promoting an immune response to TI-2 antigens in immunocompromised hosts. Although the response to the conjugates was somewhat less in nude mice than in normal immunocompetent animals, the anti-polysaccharide titer increased more than 10 fold when the individual components were combined to form the claimed conjugate. T cells have long been known to regulate the TI-2 response. In

view of the extrathymic origin of T cells in the nude mouse, and the unusual predominance of TCR $\gamma\delta$ in these animals, it is hypothesized that less dramatic response, as compared to controls, reflects some underlying unbalance in suppressive functions among those T cells remaining in the nude mouse.

As noted above, every T cell expresses a combination of either α/β or γ/δ TCR polypeptides. The failure of a developing T cell to express both members of an α/β , or γ/δ pairing results in the death of the developing cell. Thus, a homozygous null mutation of TCR β results in the complete absence of all $\alpha\beta$ T cells, whereas ablation of TCR δ gene blocks development of all $\gamma\delta$ T cells. The T cell receptor knock-out mice (TCR-KO) used in this experiment bear homozygous null mutations at both the TCR β and TCR δ loci and are thus completely devoid of T cells.

The primary response to TI-2 - lipoprotein conjugates in T cell deficient animals does not differ significantly from that seen in immunocompetent controls injected with unconjugated components. This low primary response thus appears to reflect an underlying involvement of T cells in the early stages of the response. However, the apparent requirement for T cells in eliciting a vigorous primary response to the TI-2 antigen does not extend to the secondary response.

In striking contrast to the low primary response in a T cell null background, secondary challenge with conjugates of the invention promotes extensive class switching and the increased antibody titers indicative of a vigorous secondary response. In particular, secondary challenge with Pn14-Lipo OspA in the complete absence of T cells promotes a level of anti-Pn14 antibodies comparable to that seen in immunocompetent control animals.

The relatively lower, yet still vigorous response to the Pn14-Lipo D conjugate may be a secondary effect of the poor primary response in these animals. It is predicted that subsequent booster injections will further promote the secondary responses and increase antibody titers to levels obtained in immunocompetent hosts.

Thus, not only do the compositions of the invention demonstrate the surprising and unexpected properties of promoting a vigorous immune response to TI-2 antigens, including extensive isotype switching and memory response, but the compositions also exhibit the remarkable property of promoting class switching and memory response in the complete absence of T cells.

EXAMPLE 5

Lipo OspA-Pn14 elicits a humoral immune response in subjects lacking both T cells and NK cells.

Natural killer cells, or "NK cells," are lymphoid cells that mediate certain "nonspecific" cytotoxic responses which constitute a major effector arm of self-defense in the immune system. Such nonspecific cytotoxic responses destroy certain forms of tumor cells through contact interaction, using recognition systems that are different from those used by T or B cells. In addition to their anti-neoplastic functions, it has been suggested that NK cells may be directly or indirectly activated by TI-2 antigens, and in turn, stimulate B cells exposed to those antigens. In view of the finding that the compositions of the invention promote extensive class switching and vigorous anamnestic response in the absence of T cells, and the suggestion that NK cells may play a role in the TI-2 response, we examined the humoral immune response to the conjugates of the invention in the absence of both T and NK cells.

CBA/B6F, CD3 ϵ transgenic mice (CD3 ϵ -TG) are programed to express constitutive levels of the CD3-epsilon protein in the precursor cells that give rise to NK and T cells. The effect of this ectopic expression is to block further development of all NK and T lineages resulting in an animal which constitutively lacks both cell types. In order to test whether the composition of the invention would promote class switching and memory in the absence of both NK and T cells, CBA/B6F, control and CD3 ϵ -transgenic mice were immunized subcutaneously on day 0 with 2.5 μ g of Pn14-Lipo OspA prepared as described in Example 1. Mice were bled on day 14 and

then boosted subcutaneously with an identical injection of 2.5 μ g of Pn14-Lipo OspA. Test bleeds were again taken on day 28. IgG₁ titers (ng/ml) are indicative of both class switching from the IgM isotype and the general level of humoral immune response to subcutaneous injection. Anti-Pn14 antibodies of the IgG₁ subclass were thus examined for evidence of class switching and boost response.

Table 5

The humoral immune response elicited by Lipo OspA is reduced in mice lacking both T and NK cells

genotype	bleed	animal			IgG1 anti-Pn14 titer	Mean IgG1 anti-Pn14 titer	Std Dev	Std Error					
CD3 ξ -TG	day 14	1a	0.14	40	69	37	2.21	1.42					
			0.03	160									
		1b	0.12	40	55								
			0.04	160									
		1c	0.21	10	32								
			0.08	40									
		1d	0.10	10	10								
			0.08	40									
		1e	0.12	40	60								
			0.05	160									
CBA/B6F ₁	day 14	2a	0.10	640	729	1658	2.10	1.39					
			0.05	2560									
		2b	0.17	640	1577								
			0.08	2560									
		2c	0.18	640	1496								
			0.05	2560									
		2d	0.14	2560	4400								
			0.04	10240									
		CD3 ξ -TG	day 28	1a	0.13				640	1002	514	4.16	1.89
					0.03				2560				
1b	0.17			160	409								
	0.07			640									
1c	0.21			160	567								
	0.09			640									
1d	0.19			160	470								
	0.08			640									
1e	0.14			40	77								
	0.06			160									
CBA/B6F	day 28	2a	0.13	2560	4268	19923	2.26	1.44					
			0.05	10240									
		2b	0.25	2560	5907								
				10240									

genotype	bleed	animal			IgG1 anti-Pn14 titer	Mean IgG1 anti-Pn14 titer	Std Dev	Std Error		
		2c	0.19	102	27261					
				40						
			0.06	409						
		2d		60	32131					
			0.19	102						
				40						
		2e	0.08	409	30447					
				60						
			0.18	102						
									40	
					0.08				409	
									60	
		+	0.15	160	336					
			0.06	640						
		nms	0.12	10	13					
				40						
			0.03	40						

In accord with the results obtained in the absence of T cells alone, the primary response in CD3 ξ -TG mice is extremely low. Interestingly, the secondary response in CD3 ξ -TG mice is markedly lower than in control animals, indicating that, at least in the absence of T cells, NK cells play a significant role in the secondary responses promoted by the instant invention. Notably, the mean IgG1 levels in CD3 ξ -TG mice injected with the conjugate is significantly greater than the IgG1 levels previously demonstrated when unconjugated Pn14 and Lipo OspA was injected into various strains of mice. The increased IgG1 titer may indicate that the conjugate promotes some level of class switching, memory response or both in the absence of T and NK cells, and thus suggests a role for one or more additional cell types. This relatively low IgG1 titer may reflect an inefficient primary response, as opposed to an intrinsic defect in the secondary pathway, thus, the attenuated amnestic response may be remedied by additional booster injections. Nevertheless, the clear anti-polysaccharide response in CD3 ξ -TG mice further support compositions and methods for promoting and immune response to TI-2 antigens in severely immunocompromised patients.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

We claim:

1. A composition comprising a Type 2 T cell-independent antigen conjugated to a lipid or lipid-containing moiety that promotes an immune response to the Type 2 T cell-independent antigen.
2. The composition of claim 1 wherein the lipid or lipid-containing moiety is wholly or partly synthetic.
3. The composition of claim 1 wherein the lipid or lipid-containing moiety is a lipoprotein.
4. The composition of claim 3 wherein the lipoprotein is derived from a microorganism.
5. The composition of claim 3 wherein the lipoprotein is Lipo OspA.
6. The composition of claim 3 wherein the lipoprotein is Lipo D.
7. The composition of claim 1 wherein the composition additionally comprises one or more haptens bound to the conjugate.
8. The composition of claim 1 wherein the composition further comprises one or more cytokines, including recombinant cytokine fusion proteins.
9. The composition of claim 8 wherein at least one cytokine is selected from the group consisting of IL-1, IL-2, IL-3, GM-CSF, IFN- γ .
10. The composition of claim 8 wherein at least one cytokine is selected from the group consisting of IL-4, and IL-5.
11. The composition of claim 1 wherein the composition further comprises CD40 ligand.
12. The composition of claim 1 wherein the composition further comprises one or more T dependent antigens bound to the conjugate.
13. The composition of claim 1 wherein the Type 2 T cell-independent antigen is a polysaccharide.
14. The composition of claim 13 wherein the polysaccharide is derived from a bacteria selected from a group consisting of *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, Group B *Streptococcus*, *Neisseriae meningitidis*, *Salmonella*, and *Pseudomonas aeruginosa*.

15. The composition of claim 13 wherein the polysaccharide is covalently attached to a lipoprotein via CDAP, 1-cyano-4-"dimethylamino"-pyridinium tetrafluoroborate, activation of the polysaccharide.
16. A pharmaceutical composition comprising the composition of any of claims 1-15 dissolved or suspended in a pharmaceutically acceptable carrier.
17. A vaccine comprising the composition of claims 1-15 dissolved or suspended in a pharmaceutically acceptable carrier.
18. A method of promoting an immune response to a Type 2 T cell-independent antigen in a host which comprises administering to the host a composition of claims 1-15.
19. The method of claim 18 wherein administration of the composition elicits a memory response.
20. The method of claim 19 wherein the memory response is vigorous.
21. The method of claim 19 wherein administration of the composition promotes isotype switching.
22. The method of claim 18 wherein the host is immunocompromised.
23. The method of claim 22 wherein the immunocompromised host is T cell deficient.
24. The method of claim 22 wherein the immunocompromised host is a neonate.
25. The method of claim 22 wherein the immunocompromised host is an aged individual.
26. The method of claim 22 wherein the immunocompromised host is immuno-suppressed by exposure to viruses, microorganisms, radiation, cytotoxic chemicals, corticosteroids or other immunosuppressive drugs.
27. An assay system for identifying compositions useful for stimulating isotype switching and/or the B cell memory response comprising:
 - a) administering a composition of claims 1-15 to B cells in the absence of T cells,
 - b) measuring the presence or enhancement of isotype switching and/or the B cell memory response, and
 - c) identifying or obtaining the compositions that have stimulated

28. The assay of claim 27 wherein the B cells are isolated B cells.
29. The assay of claim 28 wherein the B cells are at least 98% pure B cells.
30. The assay of claim 29 wherein the B cells are at least 99% pure B cells.
31. The assay of claim 27 wherein the Type 2 T cell-independent antigen is anti-Ig-dextran.
32. The assay of claim 31 wherein the anti-Ig-dextran is IgD.
33. The assay of claim 31 wherein the anti-Ig-dextran is IgM.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US99/05647 (22) International Filing Date: 15 March 1999 (15.03.99) (30) Priority Data: 09/039,247 16 March 1998 (16.03.98) US (71) Applicant: HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE [US/US]; Suite 600, 1401 Rockville Pike, Rockville, MD 20852 (US). (72) Inventors: MOND, James, J.; 527 Northwest Drive, Silver Spring, MD 20901 (US). SNAPPER, Clifford, M.; 10114 Kensington Parkway, Kensington, MD 20895 (US). (74) Agents: GARRETT, Arthur, S. et al.; Finnegan, Henderson, Farabow Garrett & Dunner, L. L.P., 1300 I Street, Wash- ington, DC 20005-3315 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published. <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims</i> <i>and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 23 December 1999 (23.12.99)
(54) Title: INDUCTION AND ENHANCEMENT OF THE IMMUNE RESPONSE TO TYPE-2 T CELL-INDEPENDENT ANTIGENS CONJUGATED TO LIPID OR LIPID-CONTAINING MOIETIES (57) Abstract The present invention provides a method of promoting a vigorous immune response to Type-2 T cell-independent antigens, such as bacterial polysaccharides, by the administration of a composition comprising a Type-2 T cell-independent antigen conjugated to a lipid or lipid-containing moiety, preferably, Lipo OspA. The composition promotes extensive class switching and memory to Type-2 T cell-independent antigens in immunocompetent and T cell-deficient hosts.		

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/05647

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/385 A61K39/39 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 32963 A (HENRY M. JACKSON FOUNDATION FOR THE ADVANCEMENT OF MILITARY MEDICINE) 24 October 1996 (1996-10-24) page 32 -page 33	1-26
X	LEES A ET AL: "Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate for use in protein-polysaccharide conjugate vaccines and immunological reagents" VACCINE, vol. 14, no. 3, 1 February 1996 (1996-02-01), page 190-198 XP004057339 ISSN: 0264-410X the whole document	1-26

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

21 October 1999

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INTERNATIONAL SEARCH REPORT

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PCT/US 99/05647

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 651 971 A (LEES A.) 29 July 1997 (1997-07-29) column 31 -column 32 ----	1-26
A	SNAPPER C M ET AL: "Restoration of T cell - independent type 2 induction of Ig secretion by neonatal B cells in vitro." JOURNAL OF IMMUNOLOGY, (1997 MAR 15) 158 (6) 2731-5. , XP002119809 the whole document -----	1-32

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 05647

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 18-26 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
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Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/05647

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9632963 A	24-10-1996	AU 5485696 A	07-11-1996
		CA 2218385 A	24-10-1996
		EP 0824360 A	25-02-1998
		JP 11508225 T	21-07-1999
US 5651971 A	29-07-1997	AU 5259196 A	08-10-1996
		CA 2215933 A	26-09-1996
		EP 0814833 A	07-01-1998
		JP 11502820 T	09-03-1999
		NZ 304715 A	29-07-1999
		WO 9629094 A	26-09-1996
		US 5693326 A	02-12-1997
		US 5849301 A	15-12-1998
		AU 678613 B	05-06-1997
		AU 7839194 A	10-04-1995
		CA 2171942 A	30-03-1995
		EP 0720485 A	10-07-1996
		JP 9502978 T	25-03-1997
		NZ 274376 A	24-11-1997
		WO 9508348 A	30-03-1995